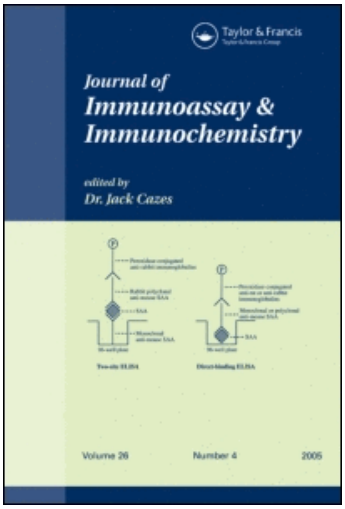

Journal of Immunoassay and Immunochemistry

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

Direct Radioimmunoassay for the Measurement of Serum Progesterone using ^{3}H as a Label

Tulsidas G. Shrivastav[a]; Pawan K. Kanaujia[b]; Ravi Kumar[c]
[a] Department of Reproductive Biomedicine, National Institute of Health and Family Welfare, Munirka, New Delhi, India [b] Department of Biotechnology, Veer Bahadur Singh Purvanchal University, Jaunpur, India [c] Department of Biotechnology, IP College, Bulandshahr, Chaudhay Charan, Singh University, Meerut, India

To cite this Article Shrivastav, Tulsidas G. , Kanaujia, Pawan K. and Kumar, Ravi(2007) 'Direct Radioimmunoassay for the Measurement of Serum Progesterone using ^{3}H as a Label', Journal of Immunoassay and Immunochemistry, 28: 2, 137 — 146
To link to this Article: DOI: 10.1080/15321810701211742
URL: http://dx.doi.org/10.1080/15321810701211742

Journal of Immunoassay & Immunochemistry, 28: 137–146, 2007

ISSN 1532-1819 print/1532-4230 online
DOI: 10.1080/15321810701211742

Direct Radioimmunoassay for the Measurement of Serum Progesterone using ^{3}H as a Label

Tulsidas G. Shrivastav
Department of Reproductive Biomedicine, National Institute of Health and Family Welfare, Munirka, New Delhi, India

Pawan K. Kanaujia
Department of Biotechnology, Veer Bahadur Singh Purvanchal University, Jaunpur, India

Ravi Kumar
Department of Biotechnology, IP College, Bulandshahr, Chaudhay Charan, Singh University, Meerut, India

Abstract: Direct radioimmunoassay (RIA), based on the principle of competitive inhibition for the measurement of serum progesterone using ^{3}H as label, is described. Progesterone 3-O-carboxymethyloxime- bovine serum albumin (progesterone 3-O-CMO-BSA) was used as an immunogen and progesterone labeled at positions 1, 2, 6, and 7 with ^{3}H was used as tracer. To 12 mm × 75 mm glass tubes, 100 μL of standard (250 pg to 50,000 pg/mL) and unknown samples were added, in duplicate, followed by 100 μL of antibody and 600 μL of tracer (10,000 counts per minute [cpm]) in all of the tubes and incubated overnight at 4°C. The bound and free fractions of labeled material were separated by adding 200 μL of charcoal followed by centrifugation. The bound radioactivity was measured in the supernatant by using a scintillation fluid containing 2,5-diphenyloxazole (PPO, primary scintillator) and p-Bis[2-(5-phenyloxazolyl)]-benzene (POPOP, secondary scintillator). In the present study, a high ionic strength, along with low and neutral pH of the buffer, is

Address correspondence to Dr. T. G. Shrivastav, Department of Reproductive Biomedicine, National Institute of Health and Family Welfare, Munirka, New Delhi 110067, India. E-mail: tgsrivastava@nihfw.org

utilized to release bound steroid from proteins. The sensitivity of the assay is 732 pg/mL. The recovery ranged between 94.03% to 100.96%. The inter-assay and intra-assay coefficients of variation ranged from 3.89% to 7.59% and from 9.96% to 12.6%, respectively. The serum progesterone values, obtained by this method, were correlated with those obtained by enzyme linked immunosorbent assay; $r = 0.96$ ($n = 94$).

Keywords: Progesterone, Direct radioimmunoassay, ^{3}H labeled immunoassay, Enzyme linked immunosorbent assay

INTRODUCTION

Progesterone, a C_{21} steroid secreted by the corpus luteum, promotes the development of the endometrial lining. Serum levels of progesterone rise during the luteal phase of the menstrual cycle. If conception occurs, levels increase dramatically from the end of the first trimester to term during pregnancy. Because progesterone is required for the maintenance of pregnancy, low levels are associated with luteal phase defect, ectopic gestation, and miscarriage. Many immunoassay techniques have been developed for measuring progesterone in serum. Most of these use different reagents or combinations of reagents as displacers in the tracer buffer or sample dilution buffer to displace the steroid from the specific steroid binding protein. Danazol, dexamethasone, and cortisol were used as displacers in the tracer buffer for measuring progesterone directly from serum.[1–3] Trichloroacetic acid, dihydrotestosterone, saponin, and isothiazolinone were used in the sample dilution buffer for the same purpose.[4,5] In the present work, instead of addition of a displacer, either in the tracer or the sample dilution buffer, we have studied the effect of high ionic strength, along with low and neutral pH of the buffer to cause the release of bound steroid from proteins.

EXPERIMENTAL

Progesterone, progesterone-3-O-carboxy-methyl-oxime (P-3-O-CMO), 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide-HCL (EDAC-HCL), N-hydroxy-succinimide (NHS), bovine serum albumin (BSA), Freund's complete adjuvant (FCA) thimerosal, activated charcoal, and dextran T-70 were all purchased from Sigma Chemical Company, St. Louis, MO, USA. Other steroids were purchased from Steraloids, Inc. Ltd., 94 Tachbrook Street, London SW 1V 2NB, England. Progesterone, with ^{3}H at positions 1, 2, 6, and 7, was purchased from NEN Life Science Products, Boston, MA, U.S.A. All other chemicals and buffer salts were purchased from Sisco Research Laboratory (SRL), Bombay, India.

Buffer

The buffers used were

a) 100 mmol/L acetate buffer, pH 4.0, (CH_3COONa, 8.2 gm/L and 7.4 mL of glacial acetic acid) containing 46 gm/L sucrose, 8.6 gm/L ammonium sulfate, 1 gm/L BSA, and 0.1 gm/L thimerosal.

b) 100 mmol/L phosphate buffer, pH 7.2, ($NaH_2PO_4 \cdot 2H_2O$ 3.86 gm/L and $Na_2HPO_4.2H_2O$ 10.2 gm/L) containing 46 gm/L sucrose, 8.6 gm/L ammonium sulfate, 1 gm/L BSA, and 0.1 gm/L thimerosal.

Immunogen Preparation

Progesterone 3-O-carboxymethyloxime (P-3-O-CMO) was conjugated to BSA according to the method of Shrivastav et al.[6] In brief, ten milligram (10 mg) of P-3-O-CMO was dissolved in 400 μL of dioxan and 400 μL of dimethyl formamide. To 200 μL of distilled water, 20 mg of N-hydroxysuccinimide (NHS) and 40 mg of 1-ethyl−3(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDAC-HCL) were dissolved; the aqueous mixture was added to the steroid solution. The reaction mixture was vortex-mixed and incubated overnight at 4°C to activate the -COOH group of the steroid. One hundred milligrams of bovine serum albumin (BSA) were dissolved in 100 mL of distilled water. The activated steroid was added slowly to the aqueous solution of BSA. The reaction mixture was further vortex-mixed and incubated overnight at 4°C. The steroid−BSA conjugate was dialyzed against distilled water containing charcoal to adsorb the free steroid coming from the dialysis bag.

The dialysate was centrifuged to remove any precipitate and was then lyophilized and kept at −30°C in a desiccator, and used for immunization.

Antibody Generation

New Zealand white rabbits were immunized with P-3-O-CMO-BSA following the method described elsewhere.[6] In brief, 1 mg of lyophilized progesterone derivative was dissolved in 0.5 mL of normal saline and mixed with 0.5 mL of Freund's Complete Adjuvant (FCA) and emulsified. The emulsion was injected intramuscularly, in equal volumes, into the thigh muscle and the shoulder blade. Intramuscular injections of progesterone-BSA conjugate emulsion were repeated on days 7, 14, and 21 following the initial injection. The booster injections were given every 30 days thereafter. Blood was regularly collected 14 days after the booster injections. Serum was separated and stored at −30°C in the deep freezer in 1 mL aliquots.

Titer

The antiserum was serially diluted with 100 mmol/L acetate buffer to give 1:500, 1:1,000, 1:2,000, 1:4,000, 1:8,000, and 1:16,000 dilutions. A volume of 100 μL of diluted antiserum was added to 12 mm × 75 mm glass tubes in duplicate, followed by the addition of radioactive [^{3}H]-progesterone (600 μL containing, 10,000 cpm), mixed, and kept overnight at 4°C. Separation of free and bound progesterone was achieved by adding 200 μL of Dextran coated charcoal suspension (0.625 gm activated charcoal and 0.0625 gm Dextran T-70 in 100 mL acetate buffer). The reaction mixture was kept for 20 min at 4°C and then centrifuged at 2,000 rpm for 10 min at 4°C. Supernatants containing the antibody bound [^{3}H]-progesterone were decanted into a scintillation vial containing 10 mL of scintillation fluid (3 gm of PPO and 0.1 gm of POPOP dissolved in one liter of toluene) and counted in a Wallac Liquid Scintillation Counter (Model 1409). The titer of the progesterone antiserum was defined as the dilution at which 50% of the tritiated progesterone was bound to the antibody.

Development of Direct Radioimmunoassay using Acetate Buffer

To 12 mm × 75 mm glass tubes, 100 μL of standard (250 pg to 5,000 pg/mL)/or unknown samples were added, in duplicate, followed by 100 μL of antibody and 600 μL of tracer (10,000 cpm) to all of the tubes and incubated overnight at 4°C. Separation of free and bound progesterone was achieved by adding 200 μL of Dextran coated charcoal suspension (0.625 gm activated charcoal and 0.0625 gm Dextran T-70 in 100 mL acetate buffer). The reaction mixture was kept for 20 min at 4°C and then centrifuged at 2,000 rpm for 10 min at 4°C. Supernatants containing the antibody bound [^{3}H]-progesterone were decanted into a scintillation vial containing 10 mL of scintillation fluid (3 gm of 2,5-diphenyloxazole (PPO, primary scintillator) and 0.1 gm of p-bis [2-(5-phenyloxazolyl)]-benzene (POPOP, secondary scintillator) dissolved in one liter of toluene], and counted in a Wallac Liquid Scintillation Counter (Model 1409).

Development of Direct Radioimmunoassay using Phosphate Buffer

To 12 mm × 75 mm glass tubes, 100 μL of standard (250 pgm to 20,000 pgm/mL)/or unknown samples were added, in duplicate, followed by 100 μL of antibody and 600 μL of tracer (10,000 cpm) to all of the tubes and incubated overnight at 4°C. Separation of free and bound progesterone was achieved by adding 200 μL of Dextran coated charcoal suspension (0.625 gm activated charcoal and 0.0625 gm Dextran T-70 in 100 mL phosphate buffer). The reaction mixture was kept for 20 min at 4°C and then centrifuged at 2,000 rpm for 10 min at 4°C. Supernatants containing

the antibody bound [^{3}H]-progesterone were decanted into a scintillation vial containing 10 mL of scintillation fluid (3 gm of 2,5-diphenyloxazole (PPO, primary scintillator) and 0.1 gm of p-bis [2-(5-phenyloxazolyl)]-benzene (POPOP, secondary scintillator) dissolved in one liter of toluene) and counted in a Wallac Liquid Scintillation Counter (Model 1409).

Enzyme Linked Immunosobent Assay (ELISA)

Progesterone was measured in the samples by ELISA as per the method of Basu et al.[7] To the progesterone antibody coated wells, 50 μL of different concentrations of standards or 50 μL of samples were added in duplicate. 100 μL of working dilution of enzyme conjugate (17α OH-P-3-O-CMO-ALP) was added to all of the wells and incubated for one hour at 37°C. After incubation, wells of the micro-titer plates were washed in running tap water. In the next step, 100 μL of pNPP substrate (1 mg/mL in 1% diethanolamine buffer, pH 11.0, containing 0.01% of $MgCl_2$, prepared just before its use) solution was added to all of the wells and incubated for 30 minutes. No stopping solution was used. The developed yellow color was directly measured by a Tecan Spectra automatic micro-well reader at 405 nm wavelength, which could measure the absorbances of all 96 wells within 8 seconds. The progesterone concentration in the individual sample was estimated by interpolation from the calibration curve, which was prepared in semi-log graph or with a microcomputer program developed using a logit-log method.

Data Analysis

Quantification of the progesterone in serum samples were performed by a in-house developed personal computer programme written in QBASIC language using a logit-log linear regression method and Mann-Whitney test was used to compare the values of samples obtained by this direct RIA and by ELISA.

RESULTS

Sensitivity

The lowest detection limit of the assay, i.e., concentration equivalent to B_0 – 2SD, was 732 pg/mL of serum after thirty-fold determination of B_0 binding.

Specificity of Antibody

Progesterone antibody had less than 0.1% cross-reaction with naturally occurring C_{27}, C_{21}, C_{19}, and C_{18} steroids, except pregnenolone (0.26%).

Table 1. Recovery of progesterone from exogenously spiked pooled serum using acetate buffer

	Progesterone added (ng/ml)	Expected (ng/ml)	Obtained (ng/ml)	Recovery (%)
Basal	0	—	9.45	—
Low	2	11.45	11.56	100.96
Medium	5	14.45	14.44	99.93
High	10	19.45	18.29	94.03

Analytical Recoveries

The ability of the assay to accurately quantify progesterone in serum samples was tested. Low, medium, and high concentrations (2.0–10 ng/mL) of progesterone were added exogenously to three fractions of pooled serum. After addition, the concentration of progesterone was determined and recovery was calculated in each fraction of serum. The recovery ranged between 94.03% and 100.96% (Table 1), and 104% to 118.14% (Table 2), when measured using acetate buffer and phosphate buffer, respectively.

Intra-Assay and Inter-Assay Variations

Table 3 indicates the precision profile of the assay. The analysis of 4 quality control sera for intra-assay ($n = 8$, replicate of each pool) gave CVs $< 7.59\%$ at all levels. The mean $\pm$ SD concentrations measured were as follows: serum A, 9.43 ± 0.43 (ng/mL); serum B, 11.56 ± 0.45 (ng/mL); serum C, 14.44 ± 0.94 (ng/mL); serum D, 18.29 ± 1.39 (ng/mL). Inter-assay CVs for these 4 sera in 5 separate assays (8 replicate of each pool) were $< 12.60\%$ at all levels. The mean $\pm$ SD values of these samples were 8.53 ± 0.85, 11.03 ± 1.39, 14.30 ± 1.43, and 18.67 ± 1.53 ng/mL.

Table 2. Recovery of progesterone from exogenously spiked pooled serum using phosphate buffer

	Progesterone added (ng/ml)	Expected (ng/ml)	Obtained (ng/ml)	Recovery (%)
Basal	0	—	4.01	—
Low	2	6.01	7.10	118.14
Medium	5	9.01	9.37	104
High	10	14.01	15.98	114.06

Table 3. Inter and intra assay coefficient of variations (CVs) for measurement of serum progesterone

Mean ± SD ng/ml	CV%
Within assay (n = 8 each)	
9.45 ± 0.43	4.45
11.56 ± 0.45	3.89
14.44 ± 0.94	6.50
18.29 ± 1.39	7.59
Between assay (n = 5 each)	
8.53 ± 0.85	9.96
11.03 ± 1.39	12.60
14.30 ± 1.42	9.93
18.67 ± 1.53	8.19

Correlation Coefficient

Progesterone values were measured in 94 serum samples by acetate buffer based direct RIA and by ELISA. Regression analysis of the samples yielded the following equation:

$$y(\text{Direct RIA}) = 1.07 \times (\text{ELISA}) - 0.82; \quad r = 0.96$$

The values obtained by these two methods were again tested by the Mann-Whitney U Test procedure to compare the two methods. They were identical, significantly ($Z = 1.81$; $p >= 0.05$).

DISCUSSION

The developed RIA for estimation of progesterone in human serum is direct and simple. Only 100 μL of serum is required and, within 2 days, the entire assay will be completed. In the present RIA procedure, the influence of ionic strength, along with pH of the buffer, have been investigated for the release of steroids bound to binding proteins.

The competition between plasma proteins and specific antibodies for analyte is a well-documented phenomenon in the area of steroid immunoassay.[8,9] Steroids are reversibly bound to proteins in serum, and steroid immunoassays are usually classified according to the method by which this binding is overcome. Assays where the analyte is separated from other serum constituents prior to immunoassay, usually by solvent extraction, can produce analytically correct results, which agree with results of methods such as mass spectrometry.[10] Direct (non-extraction) assays use

chemical blocking/displacing agents with high affinity for serum protein binding sites to reduce or, ideally, prevent serum protein binding of the analyte.

The ^{3}H and ^{125}I radionuclides are generally used in steroid RIAs. The ^{3}H tracers used in steroid RIA are non-analogue (chemically identical to the analyte), whereas ^{125}I tracers are of general structural analogue "steroid bridge label." Analogue steroid tracers are, thus, susceptible to bridge effects. All ^{3}H and ^{125}I labeled steroid analogues bind to serum proteins. The serum protein binding of ^{3}H labeled steroids were substantially reduced in all samples by addition of blocking agents, but the binding of ^{125}I labeled steroid analogue tracers in same samples were less affected. This suggests that serum protein binding of steroid analogue tracers may be a source of interference in some direct steroid immunoassays.[11]

Major steroids (e.g., cortisol/progesterone) bound in serum to corticosteroid binding globulin (CBG) may be displaced by protein binding agents such as 8-anilino-1-naphthalene sulphonic acid (8-ANS), and salicylate, by proteolytic enzyme, by low pH, or by heat treatment.[5] In addition, danazol, dexamethazone, and cortisol, which have affinities for CBG, have been used in direct assay for progesterone.[12,13] A disadvantage of these displacing agents is that, at their blocking concentrations, some of them reduce the specific binding of the antigen with the antibody.[2] In some cases, these displacing agents also cross-react with the antibody. In several cases, a mixture of different non-related steroids is also used as displacing agent in direct assays of steroids, such as testosterone and estradiol. These exogenous steroids are potential sources of cross-reaction.[11,14] The complex mixtures of these displacing agents in direct immunoassay interfere with the performance of the assay and the sensitivity is affected.[11]

To overcome the problem of cross-reaction of blocking reagents with the specific antiserum and proper displacement of analyte and tracer from serum protein, in this study, high ionic strength along with low and neutral pH of the buffer is tested to release bound steroid from proteins, which does not have any bearing on the specificity of the antiserum.

In the present study, it was observed that the ionic strength, along with pH of the buffer, has no influence on the release of steroids bound to binding proteins. The data of the present study further strengthens the possibility of absence of specific binding globulin for progesterone, as 80% of progesterone is bound to albumin and 18% to transcortin, and remaining 2% is free.[7] As the major percentage of the progesterone is non-specifically bound with albumin, it may be readily available for antibody during immunoassay without the help of a displacer or low buffer pH.

The analytical variables of the present direct RIA, especially accuracy, which is regarded as a cornerstone of the assay, are in agreement with the standardization of the method, i.e., recovery and correlation coefficient.

ACKNOWLEDGMENTS

This study was supported by the National Institute of Health and Family Welfare, New Delhi, India. We are grateful to Prof. N. K. Sethi, and Prof. K. Kalaivani for their keen interest and encouragement throughout the study.

REFERENCES

1. Ratcliffe, W.A.; Corrie, J.E.; Dalziel, A.H.; Macpherson, J.S. Direct ^{125}I-radioligand assays for serum progesterone compared with assays involving extraction of serum. Clin. Chem. **1982**, *28*, 1314–1318.
2. Carriere, P.D.; Lee, B. Direct radioimmunoassay of progesterone in bovine plasma using danazol (17-alpha-2,4-pregnadien-20-yno(2,3-d)isoxazol-17-ol) as a displacing agent. Can. J. Vet. Res. **1994**, *58*, 230–233.
3. Bernard, G.; Osher, J.; Lichter, S.; Gayer, B.; De Boever, J.; Limor, R.; Ayalon, D.; Kohen, F. The measurement of progesterone in serum by a non-competitive idiometric assay. Steroids **1995**, *60*, 824–829.
4. Dechaud, H.; Bador, R.; Claustrat, F.; Desuzinges, C.; Mallein, R. New approach to competitive lanthanide immunoassay: time-resolved fluoroimmunoassay of progesterone with labeled analyte. Clin. Chem. **1988**, *34*, 501–504.
5. Williams, G.T.; Groskopf, W.R.; Baker, H.N.; Agdeppa, P.A. Determination of steroids by competitive immunoassay. US Patent (#6,201,141). Assignee: Abbott Park, II: Abbott Laboratories, 2001.
6. Shrivastav, T.G.; Basu, A.; Kariya, K.P. One step enzyme linked immunosorbent assay for direct estimation of serum testosterone. J. Immunoassay Immunochem. **2003**, *24*, 205–217.
7. Basu, A.; Nara, S.; Chaube, S.K.; Rangari, K.; Kariya, K.P.; Shrivastav, T.G. The influence of spacer-containing enzyme conjugate on sensitivity and specificity of enzyme immunoassays for hapten. Clin. Chim. Acta **2006**, *366*, 287–292.
8. Pratt, J.J.; Wiegman, T.; Lappohn, R.E.; Woldring, M.G. Estimation of plasma testosterone without extraction and chromatography. Clin. Chim. Acta **1975**, *59*, 337–346.
9. Brock, P.; Eldre, E.W.; Woiszwillo, J.E.; Doran, M.; Schoemaker, H.J. Direct solid phase ^{125}I radioimmunoassay of serum cortisol. Clin. Chem. **1978**, *24*, 1595–1598.
10. Schioler, V.; Thode, J. Six Direct radioimmunoassays of estradiol evaluated. Clin. Chem. **1988**, *34*, 949–952.
11. Micallef, J.V.; Hayes, M.M.; Latif, A.; Ashan, R.; Sufi, S.B. Serum binding of steroid tracers and its possible effects on direct steroi immunoassay. Ann. Clin. Biochem. **1995**, *32*, 566–574.
12. Ratcliffe, W.A. Direct (non-extraction) serum assays for steroids. In *Immunoassays for Clinical Chemistry*; Hunter, W.M., Corrie, J.E.T., Eds.; Churchill Livingstone: Edinburgh, U.K., 1983; 401–409.

13. Barnard, G.; Read, G.F.; Collins, W.P. Immunoassay. In *Steroid Analysis*; Makin, H.L.J., Gower, D.B., Kirk, D.N., Eds.; Blakie Academic and Professional (an imprint of Chapman and Hall): U.K., 1995; 185–228.
14. De Boever, J.; Kohen, F.; Usanachitt, C.; Vandekerckhove, D.; Leyseele, D.; Vanderwaalle, U. Direct chemiluminescence immunoassay for estradiol in serum. Clin. Chem. **1986**, *32*, 1895–1900.

Received September 9, 2006
Accepted October 22, 2006
Manuscript 3214